Australian Government
Department of Defence
Defence Science and
Technology Organisation

Time- and Concentration-Dependent Cytotoxicity of Ricin in Human Lung Epithelial Cells

Sharmaine Ramasamy and David Proll

Human Protection and Performance Division
Defence Science and Technology Organisation

DSTO-TR-2024

ABSTRACT

Ricin, a potent ribosome-inactivating hetero-dimeric protein toxin (66kDa) produced in the seeds of the castor bean plant (Ricinus communis), is a Category B Agent on the Centres for Disease Control (CDC) Select Agent List. Using human small airway epithelial cells, this is the first study to investigate the time- and dose-dependent cytotoxic effects of ricin in a human cell line. Ricin (1-100pM) produced a time- and dose-dependent decrease in small airway epithelial cell survival. Ricin (10 and 100pM) reduced cell survival to 57% and 50% respectively, after 12 hours exposure and to 12% cell survival after 24 hours exposure to the toxin. Washing cells after 1-5 minutes exposure to ricin (0.01-100pM) prevented any significant ricin-induced SAE cell death. However, after washing cells exposed to ricin for 15 or 30 minutes, there was a significant reduction in SAE cell survival (84% and 71%, respectively, for 10pM and 45% and 31%, respectively, for 100pM). The results suggest that the binding of ricin to the cell surface and subsequent intracellular uptake and cell death appears to be irreversible by washing between 5 and 15 minutes following exposure to the toxin. There may be a window of opportunity for treatment, and therefore prevention, of some lung cell death up to 12 hours after aerosol exposure to ricin given that there was some survival of human lung cells after this time of exposure.

RELEASE LIMITATION

Approved for public release

Published by

Human Protection and Performance Division
DSTO Defence Science and Technology Organisation
506 Lorimer St
Fishermans Bend, Victoria 3207 Australia

Telephone: (03) 9626 7000
Fax: (03) 9626 7999

AR-013-806
July 2007

APPROVED FOR PUBLIC RELEASE

Time- and Concentration-Dependent Cytotoxicity of Ricin in Human Lung Epithelial Cells

Executive Summary

Ricin is a potent protein toxin produced in the seeds of the castor bean plant. The production of ricin is a relatively simple and inexpensive process which requires the use of non-specialised equipment. The ease of production coupled with the widespread availability of the raw material, make it a biological threat agent. In addition, the delay in the onset of symptoms and a lack of specific treatments necessitate further elucidation of the mechanisms involved in ricin intoxication to assist the development of improved therapeutic countermeasures for this toxin.

There have been over 750 documented cases of ricin intoxication in humans with the vast majority of clinical cases involving ricin poisoning via castor bean ingestion. However, in the case of deliberate dissemination of ricin, dispersal by aerosol is considered to pose the greatest threat. Clinical symptoms following the inhalation of large amounts of ricin include difficulty breathing, fever, cough, nausea, pain of the joints and tightness in the chest. Victims may also suffer heavy sweating, have fluid in their lungs and their skin may have a purple tinge due to deficient oxygenation of the blood. Death may occur 24-72 hours later due to fluid accumulation in the lungs or respiratory failure, either with or without low blood pressure and organ failure. If death does not occur within 2-3 days, the patient will normally make a slow recovery (Balint, 1974; Rauber *et al.*, 1985; Challoner *et al.*, 1990; Bradberry, 2003).

To date, there is no therapy available for the direct treatment of ricin intoxication. The only indicated treatment is that of supportive medical care, including (as required) artificial respiration, administration of medications against seizures and low blood pressure, and replacement of electrolytes and fluids (in the case of severe diarrhoea and/or vomiting). Furthermore, although there have been a number of attempts to produce a ricin vaccine, there are none approved for human use.

In the present study, ricin produced both time- and concentration-dependent cytotoxicity of human lung cells. The cell death seen after 12 hour exposure to the toxin was not as great as that seen following 18-24 hour exposure to ricin. Given that there was some survival of human lung cells after the 12 hour exposure to ricin, there may be a treatment window available of up to 12 hours following aerosol exposure to ricin to prevent and/or inhibit further lung cell death. The binding of ricin to the cell surface and the consequent cascade of intracellular events that trigger the death of some human lung cells appears to be irreversible by washing after 15 minutes exposure to the toxin. This suggests that after this exposure time some lung cell death is inevitable. Collectively, these findings not only highlight the difficulties associated with treating ricin poisoning, but also the need to develop therapies that target inhibition of the

initial binding of ricin to the cell surface, as well as the cytotoxic effects of ricin that lead to cell death once it has been internalised.

The cytotoxicity assay used in the present technical report offers a simple and reproducible means of investigating the cytotoxic effects of ricin without the use of animals. In addition, other biological toxins and toxic chemicals may also be tested using this assay. A greater understanding of the mechanism(s) of the action of toxins of biosecurity importance will pave the way for the development of new therapeutics or treatment regimes and improvement of current medical support.

Authors

Sharmaine Ramasamy
Human Protection and Performance Division

Sharmaine Ramasamy graduated from Monash University in 2000 with a BA/BSc (Hons) and completed a PhD four years later in the department of Pharmacology. The work undertaken in her thesis involved a biochemical and pharmacological investigation of a number of Australasian animal venoms and testing of the efficacy of current and potential treatments and therapeutic regimes following envenoming. In 2005 Sharmaine worked as a Health Economics Associate with Kendle Pty Ltd. before returning to research and joining DSTO as a Defence Scientist later that year. Her current research with the Human Protection and Performance Division investigates the cytotoxicity of toxins and potential treatments for intoxication.

David Proll
Human Protection and Performance Division

David Proll graduated from Monash University in 1993 with a B.Sc(Hons) and went on to complete his PhD in the department of Microbiology. His PhD studies focused on the replication of positive strand RNA viruses. After graduating from university he worked at the Eijkman Institute of Molecular Biology in Jakarta, Indonesia. Here, he investigated the application and development of DNA based vaccines against the parasite that causes Malaria. Upon returning to Australia in 2000 he was recruited to DSTO to initiate a research program investigating DNA vaccines for defence applications. In 2004 he was promoted to a Senior Research Scientist and currently manages the Human Protection & Performance Divisions - Medical Countermeasures research program.

Contents

1. Introduction

Ricin, a potent protein toxin (66kDa) produced in the seeds of the castor bean plant (*Ricinus communis*), constitutes 5-10% of the total protein content of the seeds (O'Connell *et al.*, 2002). During the industrial production of castor oil, *Ricinus* seeds are pressed and the oil is extracted, leaving a ricin-rich pulp waste. It is estimated that more than one million tons of *Ricinus* seeds are processed globally each year (Mistry, 2004). The ease of production, requirement of limited specialised tools coupled with the wide-spread availability of the raw material and its extreme cytotoxicity, make it a biological threat agent. Indeed, recent links between the toxin and non-research groups in both the USA (Gibson, 2003) and the UK (BBCNews, 2003) are evidence of its production for non-scientific purposes.

Ricin is a ribosome-inactivating hetero-dimer consisting of a 32-kD A-chain that is disulfide-bonded to a 32-kD B-chain (Robertus *et al.*, 1987). The B-chain is a lectin that binds to terminal galactose residues on cell surface components and mediates the binding and entry of the holotoxin (that is, the A- and B-chain complex) into cells (Lord *et al.*, 2003), thereby promoting endocytosis and trafficking of ricin from early endosomes to the trans-Golgi network (Bigalke *et al.*, 2005). Once inside the cell, the ricin A-chain uses its endonuclease activity to inhibit protein synthesis (Greenfield *et al.*, 2002). Ricin can also bind to mannose receptors and mannose receptor-mediated uptake of this toxin has been shown to occur in macrophages (Simmons *et al.*, 1996) and rat liver endothelial cells (Magnusson *et al.*, 1993).

It is widely accepted that the toxicity of ricin results from the inhibition of protein synthesis. However, direct cell membrane damage, alteration of membrane structure and function, activation of apoptosis pathways and release of cytokine inflammatory mediators have also been demonstrated (Day *et al.*, 2002; Griffiths *et al.*, 1987; Hegde *et al.*, 1992; Hughes *et al.*, 1996; Kumar *et al.*, 2003; Lombard *et al.*, 2001; Morlon-Guyot *et al.*, 2003; Pappenheimer *et al.*, 1974). In addition to the toxin ricin, castor bean plants also produce another glycoprotein lectin, ricin communis agglutinin, which is not directly cytotoxic but does have an affinity for red blood cells and can lead to agglutination and subsequent hemolysis following intravenous administration (Balint, 1974; Hegde *et al.*, 1992; Olsnes *et al.*, 1975).

The toxicity of ricin is species-dependent and varies according to the dose and route of administration (Wilhelmsen *et al.*, 1996). In mice, a 3-5µg/kg inhalational dose of ricin killed 50% of animals within 60 hours in contrast to a 20mg/kg ingested dose which killed 50% of mice in 85 hours. The low oral toxicity reflects the limited absorption and gastrointestinal digestion of ricin (Balint, 1974). Results from non-human primates demonstrated a 24 hour delayed onset of action following inhalation exposure to ricin. All rhesus monkeys died or were killed 36-48 hours after exposure and all displayed significant lung damage, including multi-focal to coalescing fibrinopurulent pneumonia, diffuse necrosis and acute inflammation of airways, with death resulting from asphyxiation following massive pulmonary alveolar flooding (Wilhelmsen *et al.*, 1996). The principal target of ricin-induced toxicity following inhalation exposure are the type I and II

pneumocytes (Brown *et al.*, 1997; Griffiths *et al.*, 1995; Wilhelmsen *et al.*, 1996). As evidenced by a number of animal studies (Doebler *et al.*, 1995; Griffiths *et al.*, 1995; Roy *et al.*, 2003), there also appears to be no significant systemic absorption of ricin following inhalation exposure and toxicity is mainly limited to the respiratory tract. In addition, it should be noted that the lung deposition and lethality following ricin inhalation is significantly influenced by particle size, with smaller particles (i.e. those that are micron-sized) depositing much deeper in the respiratory tract and consequently causing a higher incidence of death than larger particles (Griffiths *et al.*, 1995).

To date, there have been over 750 documented cases of ricin intoxication in humans (Vitetta *et al.*, 2006) with the vast majority of clinical cases reporting ricin poisoning via castor bean ingestion (Audi *et al.*, 2005). The lethal oral dose in humans is estimated to be 1-20mg of ricin per kilogram of body weight (approximately 8 beans) (Audi *et al.*, 2005). However, in the case of deliberate dissemination of ricin, dispersal by aerosol is considered to pose the greatest threat. Clinical symptoms seen following the inhalation of significant amounts of ricin include dyspnea (difficulty breathing), fever, cough, nausea, arthralgia (pain of the joints) and tightness in the chest. Further, victims may become cyanotic with the manifestations of heavy sweating and pulmonary oedema (Challoner *et al.*, 1990; Furbee *et al.*, 1997; Kinamore *et al.*, 1980; Koch, 1942; Kopferschmitt *et al.*, 1983; Malizia *et al.*, 1977; Meldrum, 1900; Palatnick *et al.*, 2000; Rauber *et al.*, 1985; Reed, 1998; Satpathy *et al.*, 1979; Wedin *et al.*, 1986). Finally, death may occur 24-72 hours later as a consequence of high permeability pulmonary oedema, acute respiratory distress syndrome or respiratory failure, either with or without hypotension (Bigalke *et al.*, 2005; Kortepeter *et al.*, 2001) and organ failure (Franz *et al.*, 1997). If death does not occur within 2-3 days, the victim will normally make a slow recovery. To date, there is no therapy available for the direct treatment of ricin poisoning. The only indicated treatment is supportive medical care to minimise the effects of the intoxication, which is largely dependent upon the route of exposure. Medical attention may include artificial respiration, administration of medications against seizures and hypotension, and replacement of electrolytes and fluids (in the case of severe diarrhoea and/or vomiting) (Bigalke *et al.*, 2005).

Although there have been a number of attempts to produce a ricin vaccine, the majority of these efforts have been hampered by safety concerns arising from residual toxicity of native material after formaldehyde treatment. Consequently, there is currently no ricin vaccine approved for use in humans. A recent pilot study investigating the safety of a new recombinant, genetically modified ribotoxin-A vaccine (RiVax) in a small group of healthy human volunteers found that all 5 candidates who received the high dose of RiVax (100µg once per month for 3 months) produced ricin-neutralising antibodies with minimal side effects (Vitetta *et al.*, 2006). However, the protective effects of this vaccine against ricin inhalation are unknown.

This study investigates the toxicity of ricin in human small airway epithelial (SAE) cells. It aims to elucidate the binding time of ricin to the cell surface and the time to death of SAE cells, with regard to ricin concentration and time of exposure. These findings will consequently provide insight into the treatment window available following inhalational exposure to ricin. The use of these cells in an *in vitro* assay provides a simple, clinically

relevant, non-invasive and reproducible means of testing the toxicity of ricin without the use of animals. The cells will also provide a medel system for developing and evaluating potential therapeutic components.

2. Methods and Materials

2.1 Materials

Normal human small airway epithelial (SAE) cells (1st passage) and basal serum-free growth medium and growth factors were obtained from Cambrex BioScience Australia. Trypsin-EDTA, ricin (RCA_{60}), neutral red (NR) in phosphate-buffered saline (PBS – cell culture tested) and dimethyl sulfoxide (DMSO – ACS reagent) were all obtained from Sigma-Aldrich Australia. Tissue-culture-treated 96-well microplates were obtained from Costar® (product #3596).

2.2 Methods

2.2.1 Cell culture of SAE cells

Cells were cultured in a serum-free complete medium (SAGM) prepared from basal medium and growth factors. Preparation of 2nd or 3rd passage cells was initiated by thawing vials of 1st passage cells in a water bath (37°C). Aliquots of $1x10^6$ cells were resuspended in 15ml of SAGM in 75cm² vented culture flasks and incubated in an atmosphere of 5% CO_2 at 37°C. The medium was exchanged for fresh SAGM every 2-3 days.

The cells were harvested when ~80% confluent by washing with PBS, exchanging the medium for trypsin-EDTA and incubating cells at 37°C for 5-7 minutes. When cells were detached, trypsin activity was slowed by adding an equivalent volume of SAGM in a 1:1 ratio. Cell suspensions were centrifuged (200g-force, 5 minutes) and the pelleted cells were re-suspended in 5ml SAGM and re-centrifuged. For the seeding of cells in 96-well microplates, cells were then re-suspended in the appropriate volume of SAGM to allow 100µl aliquots (each containing ~$1x10^4$ cells/well) to be dispensed as required.

2.2.2 Exposure of SAE cells to ricin

Aliquots of ricin stock solution were diluted in SAGM and sterilised by passage through a 0.22µm Millex® filter. Cells were exposed to 100µl of ricin medium (0.01-100pM/well) for the given time period (1 minute – 24 hours) before being washed three times with sterile PBS. Where cells were washed after a 1-30 minutes binding time to establish the time taken for ricin to bind to the cell surface, wells were then re-filled with fresh SAGM and cells were incubated for a further 24 hours before the NR assay was performed. In the case of cells exposed to ricin for 2-24 hours to establish the time to ricin-induced cell death, NR was added to wells and the NR assay performed immediately after washing with PBS.

2.2.3 Preparation of medium for NR assays

On the day of use, NR (50µg/ml) was prepared in SAGM and incubated at 37ºC for at least 15 minutes before being added to wells.

2.2.4 Measurement of cell viability using NR uptake

Uptake of NR into viable cells was measured after cells were incubated in the NR medium for three hours. Cells were washed twice with PBS prior to the extraction of NR with 0.3% v/v HCl (1M) in DMSO for one hour. The absorbance was measured at 540nm (TiterTek MS212) and results were expressed as a % of the control (no ricin).

2.2.5 Statistics

Using the SigmaStat program, a paired t-test was performed on the mean absorbance data (expressed as % of the control) obtained for each ricin concentration (0.01-100pM) and time point (1 minute to 24 hours) performed. Differences were considered significant where a p-value < 0.05 was obtained.

3. Results

3.1 Effect of ricin (0.01-100pM) on SAE cell survival: determination of time for ricin-induced cell death

Actively proliferating SAE cells grown in tissue culture were exposed to a series of ricin concentrations (0.01-100pM) over a range of time periods (2-24 hours). After the given exposure time, excess, unbound ricin was removed by washing cells with PBS and the neutral red assay was conducted immediately. Results indicated that ricin (0.01-100pM) did not have any significant effect on SAE cell survival following a two hour exposure to the toxin (n=4; Figure 1). This means that there was no detectable ricin-induced SAE cell death after a two hour exposure to ricin. Therefore, for the purposes of these experiments, the concentration-response curve for ricin-induced SAE cell survival following a two hour exposure is not detectable and this curve may be used as the control comparator for ricin-induced death occurring at other exposure times. This gives us an indication of human lung cell survival (if any) at longer exposure times.

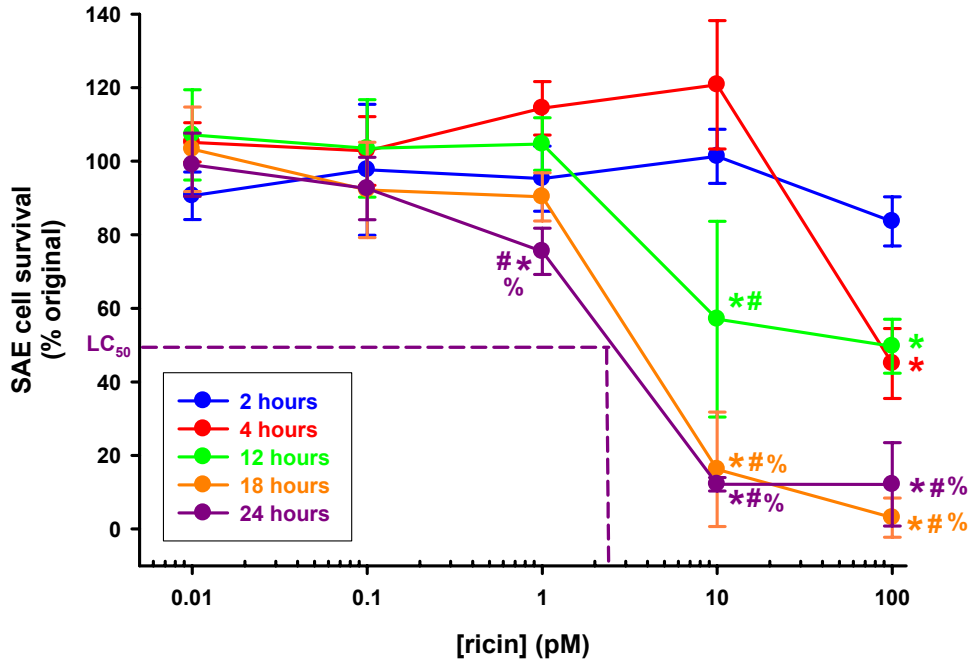

Figure 1: Assessment of the time to SAE cell death following ricin exposure.
Effect of ricin (0.01-100pM) exposure (2-24 hours) on SAE cell survival (% of original). SAE cells were cultured in serum-free complete medium in 75cm³ vented culture flasks and incubated in an atmosphere of 5% CO_2 at 37˚C. Cells were harvested when ~80% confluent and seeded onto 96 well plates. Cells were washed after the ricin exposure (2-24 hours) and the neutral red assay was performed immediately.
**Significantly different from 2 hour exposure to ricin (n=4).*
#Significantly different from 4 hour exposure to ricin (n=4).
%Significantly different from 12 hour exposure to ricin (n=4).

Ricin (1-100pM) produced a time- and dose-dependent decrease in SAE cell survival after 24 hour exposure to the toxin (n=4; Figure 1). After this time period, 75.5 ± 7.3% of SAE cells survived exposure to ricin (1pM), compared with only 12.2 ± 5.3% and 12.1 ± 11.3% of SAE cells surviving exposure to higher concentrations of ricin (10 and 100pM, respectively; n=4; $p<0.05$; Figure 1). At these concentrations of ricin, cell death was significantly greater after 18 (16.3 ± 15.5% and 3.09 ± 1.8% of control, respectively; n=4; $p<0.05$; Figure 1) and 24 hours compared with that seen at the same concentrations following a 12 hour exposure (57.1 ± 6.6% and 49.7 ± 6.3% of original, respectively) to the toxin ($p<0.05$; Figure 1). After 4 hours, ricin (100pM) significantly reduced cell survival to 45.0 ± 8.5% of the original ($p<0.05$; Figure 1), but did not have an observable effect on SAE cell survival at the lower toxin concentrations tested (0.01-10pM). In addition, cells exposed to ricin (≤0.1pM) survived 24 hour exposure to the toxin and significant cell death manifested with 1pM of ricin after 24 hours. Therefore, 24 hour post-exposure to ricin was selected as the standard time-point for subsequent assessments.

3.2 Effect of washing on SAE cell survival following exposure to ricin (0.01-100pM): determination of time for ricin to bind to cell surface

Actively proliferating SAE cells grown in tissue culture were exposed to a series of ricin concentrations (0.01-100pM) over a range of time periods (1-30 minutes). After the given binding time, excess, unbound ricin was removed by washing cells with PBS and the cells incubated in fresh SAGM for a further 24 hours prior to the neutral red assay being conducted. Results indicated that ricin (0.01-100pM) did not have any effect on SAE cell death following a one or five minute binding time to the toxin with subsequent washing with PBS (n=4; Figure 2). This means that there was no detectable ricin-induced SAE cell death after a one minute binding time to ricin. Therefore, for the purposes of these experiments, the concentration-response curve for ricin-induced SAE cell survival following a one minute binding time is used as the control comparator for ricin-induced death occurring at other binding times.

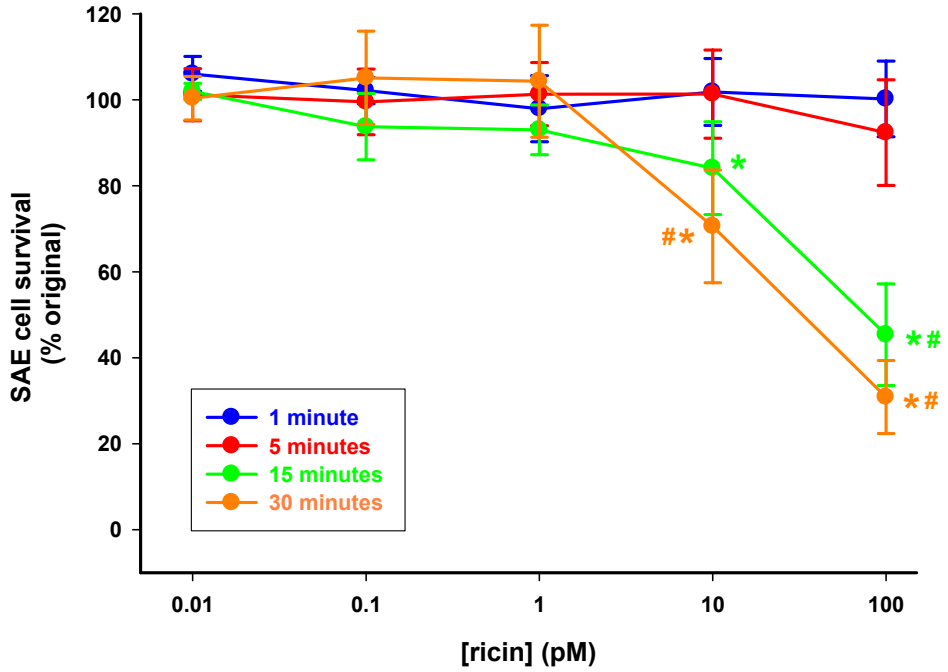

Figure 2: Determination of the time for ricin to bind to SAE cell surface.
Effect of ricin (0.01-100pM) exposure (1-30 minutes) followed by washing (3 times with PBS) on SAE cell survival (% of original). SAE cells were cultured in serum-free complete medium in 75cm³ vented culture flasks and incubated in an atmosphere of 5% CO_2 at 37˚C. Cells were harvested when ~80% confluent and seeded onto 96 well plates. Cells were washed after the ricin binding time (1-30 minutes) and incubated with fresh medium for a further 24 hours prior to performing the neutral red assay.
**Significantly different from 1 minute exposure to ricin (n=4).*
#Significantly different from 5 minute exposure to ricin (n=4).

Despite washing cells and removing excess, unbound toxin from contact with SAE cells, ricin (10-100pM) produced a time- and dose-dependent decrease in SAE cell survival after 15 and 30 minute binding times to the toxin (Figure 2). Ricin (10pM) reduced SAE cell survival to 84.1 ± 10.8% and 70.6 ± 13.1% following 15 and 30 minute binding times, respectively ($p<0.05$; n=4; Figure 2). The highest concentration of ricin (100pM) tested produced significantly greater SAE cell death at the 15 and 30 minute binding times (45.4 ± 11.8% and 30.8 ± 8.5%, respectively), compared with the control ($p<0.05$; n=4; Figure 2). The extent of ricin (0.01-100pM)-induced SAE cell death after a 30 minute binding time was not significantly greater than that seen following a 15 minute binding time.

4. Discussion

Exposure to ricin, particularly via aerosol inhalation, is of medical significance given its potential to be used as a biological warfare agent and the only treatment currently available is that of supportive care. This is the first reported study to investigate the cell surface binding time and cytotoxicity (that is, time to death) of ricin, including dose and exposure time, in a human lung epithelial cell line.

Previous studies have shown a latent period prior to the observation of symptoms of ricin poisoning in humans (Godal *et al.*, 1984) and in animals (Fodstad *et al.*, 1979; Fodstad *et al.*, 1976; Ishiguro *et al.*, 1992; Wilhelmsen *et al.*, 1996). Indeed, animals exposed to inhalational ricin display a dormant asymptomatic period of approximately 8 hours after which necrosis of upper and lower respiratory epithelium, leading to tracheitis, bronchitis, bronchiolitis, and interstitial pneumonia with perivascular and alveolar oedema can be seen (Kortepeter *et al.*, 2001). These results confirm those of other animal studies which suggest that there can be a delay in the systemic distribution of ricin to some parts of the body, coupled with a delay in the onset of symptoms, following different routes of administration of ricin (Ramsden *et al.*, 1989; Roy *et al.*, 2003). Similarly, prolonged ricin-induced cell death has been shown in other *in vitro* studies using different cell lines, including bovine pulmonary endothelial cells (Hughes *et al.*, 1996) and hepatoma cells (BEL7404) (Hu *et al.*, 2001). Therefore, the latent onset of ricin-induced SAE cell death, and the increased SAE cell death at longer exposure times to ricin are not unexpected in the present study. Indeed, further elucidation of the latent action of ricin, including the time taken for ricin to bind to the cell surface, be internalised and consequently cause cell death, will aid in establishing a potential treatment window in which therapies may be used to prevent or reduce the cytotoxic effects of ricin.

Ricin produces time-and dose-dependent death of human SAE cells, as evidenced by the results of the present study that show the significantly greater degree of cell death following a 12-24 hour exposure to ricin compared with shorter time frames (2-4 hours). This is consistent with previous studies performed in our laboratory (Paddle *et al.*, 2003) and in other publications (Griffiths *et al.*, 1994; Hassoun *et al.*, 1999; Sharma *et al.*, 1999). Further, given that there is significantly greater SAE cell death after 12 hour exposure to

the toxin compared with the 2-4 hour exposure, there may be a window of opportunity for treatment, and therefore minimisation and/or prevention, of some lung cell death up to 12 hours after exposure to ricin.

Once the ricin molecule has bound to cell surface receptors and triggered its endocytotic uptake into the cell, it would appear that the mechanism(s) set in place for ricin-induced cell death are unable to be reversed. Despite washing cells repeatedly to remove excess, unbound ricin, the present study demonstrated that there was significant death of SAE cells after 15 and 30 minute binding times of the toxin. Further elucidation of the fate of ricin once internalisation has occurred is required in order to determine where therapeutics may be targeted in order to neutralise this toxin once it has crossed the cell membrane. Histological research is currently underway to clarify where ricin may be intercepted in order to prevent cell death and consequently, which therapeutics would be the most useful in overcoming ricin intoxication.

The neutral red assay is a quick and simple method which has been widely used with a variety of cell lines to determine the cytotoxicity of various toxins and toxic agents (Babich *et al.*, 1991; Cardona *et al.*, 2006; Fotakis *et al.*, 2006; Hughes *et al.*, 1996; Valdivieso-Garcia *et al.*, 1993; Zhang *et al.*, 1990). However it must be acknowledged that there is a limitation in drawing conclusions from this *in vitro* study. The assay works on the premise that only live cells are capable of accumulating the neutral red dye in lysosomes. It does not account for cells which are able to take up the neutral red at the time the dye is added (that is cells that are alive), but will consequently die after the conclusion of the experiment due to the internalisation of the toxin. Therefore, we can not entirely account for the lethal effects of ricin once the toxin has been internalised and the cascade of events leading to cell death have been triggered. This not only highlights the difficulties associated with treating ricin poisoning, but also the need to develop antibodies and/or other treatments targeted at inhibiting the initial binding of ricin to the cell surface in addition to the cytotoxic effects of ricin that lead to cell death once it has been internalised. These difficulties are compounded by the slow degradation of ricin by cells and the ability of ricin to remain intact and active after it has been released by a cell that it has already destroyed (Sandvig *et al.*, 1978). Further elucidation of the mechanism(s) of action of ricin using *in vitro* methods will facilitate the development and assessment of therapies for the prevention and treatment of ricin intoxication.

In contrast to the study performed by Griffiths et al (1994) which found that a lethal dose of ricin (isolated from the beans of *Ricinus communis*) of 5.1pM was required to kill 50% of bovine pulmonary endothelial cells within 24 hours (LC_{50}), the LC_{50} of ricin in human SAE cells was at least half that at approximately 2.5pM. This finding indicates a higher sensitivity of human lung cells to ricin compared with bovine pulmonary endothelial cells and supports previous studies which indicate species differences to the toxicity of ricin (Balint, 1974; Sandvig *et al.*, 1978) and emphasises the importance of using human cell lines in cytotoxicity assays, where possible, rather than those derived from animals. The use of clinically relevant human cell lines and protocols will advance our understanding of the mechanism of action of ricin and undoubtedly strengthen our capabilities to prepare, as well as respond, to an incident which may occur involving this toxic biological agent. It

will also enable further examination and development of medical countermeasures to toxin exposure to be undertaken.

5. Acknowledgements

We would like to thank Dr Brian Paddle for his advice and guidance with the neutral red assay.

6. References

Audi, J., Belson, M., Patel, M., Schier, J. & Osterloh, J. (2005). Ricin poisoning: A comprehensive review. *JAMA*, 294, 2342-2351.

Babich, H. & Borenfreund, E. (1991). Cytotoxicity of T-2 toxin and its metabolites determined with the neutral red cell viability assay. *Appl Environ Microbiol*, 57, 2101-3.

Balint, G.A. (1974). Ricin: the toxic protein of castor oil seeds. *Toxicology*, 2, 77-102.

BBCNews (2003). Terror police find deadly poison.

Bigalke, H. & Rummel, A. (2005). Medical aspects of toxin weapons. *Toxicology*, 214, 210-20.

Brown, R.F. & White, D.E. (1997). Ultrastructure of rat lung following inhalation of ricin aerosol. *Int J Exp Pathol*, 78, 267-76.

Cardona, P.J., Soto, C.Y., Martin, C., Giquel, B., Agusti, G., Guirado, E., Sirakova, T., Kolattukudy, P., Julian, E. & Luquin, M. (2006). Neutral-red reaction is related to virulence and cell wall methyl-branched lipids in Mycobacterium tuberculosis. *Microbes Infect*, 8, 183-90.

Challoner, K.R. & McCarron, M.M. (1990). Castor bean intoxication. *Ann Emerg Med*, 19, 1177-83.

Day, P.J., Pinheiro, T.J., Roberts, L.M. & Lord, J.M. (2002). Binding of ricin A-chain to negatively charged phospholipid vesicles leads to protein structural changes and destabilizes the lipid bilayer. *Biochemistry*, 41, 2836-43.

Doebler, J.A., Wiltshire, N.D., Mayer, T.W., Estep, J.E., Moeller, R.B., Traub, R.K., Broomfield, C.A., Calamaio, C.A., Thompson, W.L. & Pitt, M.L. (1995). The distribution of [^{125}I]-ricin in mice following aerosol inhalation exposure. *Toxicology*, 98, 137-49.

Fodstad, O., Johannessen, J.V., Schjerven, L. & Pihl, A. (1979). Toxicity of abrin and ricin in mice and dogs. *J Toxicol Environ Health*, 5, 1073-84.

Fodstad, O., Olsnes, S. & Pihl, A. (1976). Toxicity, distribution and elimination of the cancerostatic lectins abrin and ricin after parenteral injection into mice. *Br J Cancer*, 34, 418-25.

Fotakis, G. & Timbrell, J.A. (2006). In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicol Lett*, 160, 171-7.

Franz, D. & Jaax, N. (1997). Ricin toxin. In *Medical Aspects of Chemical and Biological Warfare*. eds Sidell, F.R., Takafuji, E.T. & Franz, D.R. pp. 631-642. Falls Church, Virginia: Office of the Surgeon General.

Furbee, B. & Wermuth, M. (1997). Life-threatening plant poisoning. *Crit Care Clin*, 13, 849-88.

Gibson, J. (2003). Investigation of a ricin-containing envelope at a postal facility South Carolina. *Morb Mortal Wkly*, 52, 1129-1131.

Godal, A., Fodstad, O., Ingebrigtsen, K. & Pihl, A. (1984). Pharmacological studies of ricin in mice and humans. *Cancer Chemother Pharmacol*, 13, 157-63.

Greenfield, R.A., Brown, B.R., Hutchins, J.B., Iandolo, J.J., Jackson, R., Slater, L.N. & Bronze, M.S. (2002). Microbiological, biological, and chemical weapons of warfare and terrorism. *Am J Med Sci*, 323, 326-40.

Griffiths, G.D., Leek, M.D. & Gee, D.J. (1987). The toxic plant proteins ricin and abrin induce apoptotic changes in mammalian lymphoid tissues and intestine. *J Pathol*, 151, 221-9.

Griffiths, G.D., Lindsay, C.D. & Upshall, D.G. (1994). Examination of the toxicity of several protein toxins of plant origin using bovine pulmonary endothelial cells. *Toxicology*, 90, 11-27.

Griffiths, G.D., Rice, P., Allenby, A.C., Bailey, S. & Upshall, D.G. (1995). Inhalation toxicology and histopathology of ricin and abrin toxins. *Inhalation Toxicology*, 7, 269-288.

Hassoun, E. & Wang, X. (1999). Time- and concentration-dependent production of superoxide anion, nitric oxide, DNA damage and cellular death by ricin in the J774A.1 macrophage cells. *Journal of Biochemical and Molecular Toxicology*, 13, 179-185.

Hegde, R. & Podder, S.K. (1992). Studies on the variants of the protein toxins ricin and abrin. *Eur J Biochem*, 204, 155-64.

Hu, R., Zhai, Q., Liu, W. & Liu, X. (2001). An insight into the mechanism of cytotoxicity of ricin to hepatoma cell: roles of Bcl-2 family proteins, caspases, Ca^{2+}-dependent proteases and protein kinase C. *J Cell Biochem*, 81, 583-93.

Hughes, J., Lindsay, C. & Griffiths, G. (1996). Morphology of ricin and abrin exposed endothelial cells is consistent with apoptotic cell death. *Human and Experimental Toxicology*, 15, 443-451.

Ishiguro, M., Tanabe, S., Matori, Y. & Sakakibara, R. (1992). Biochemical studies on oral toxicity of ricin. IV. A fate of orally administered ricin in rats. *J Pharmacobiodyn*, 15, 147-56.

Kinamore, P.A., Jaeger, R.W. & de Castro, F.J. (1980). Abrus and ricinus ingestion: management of three cases. *Clin Toxicol*, 17, 401-5.

Koch, L. (1942). Castor bean poisoning. *AJDC*, 64, 485-486.

Kopferschmitt, J., Flesch, F., Lugnier, A., Sauder, P., Jaeger, A. & Mantz, J.M. (1983). Acute voluntary intoxication by ricin. *Hum Toxicol*, 2, 239-42.

Kortepeter, M., Christopher, G. & Cieslak, T. (2001). *USAM-RIID's medical management of biological casulaties handbook*. Fort Detrick, MD: US Army Medical Reseach Institute of Infectious Diseases.

Kumar, O., Sugendran, K. & Vijayaraghavan, R. (2003). Oxidative stress associated hepatic and renal toxicity induced by ricin in mice. *Toxicon*, 41, 333-8.

Lombard, S., Helmy, M. & Pieroni, G. (2001). Lipolytic activity of ricin from *Ricinus sanguineus* and *Ricinus communis* on neutral lipids. *Biochemical Journal*, 358, 773-781.

Lord, M.J., Jolliffe, N.A., Marsden, C.J., Pateman, C.S., Smith, D.C., Spooner, R.A., Watson, P.D. & Roberts, L.M. (2003). Ricin. Mechanisms of cytotoxicity. *Toxicol Rev*, 22, 53-64.

Magnusson, S. & Berg, T. (1993). Endocytosis of ricin by rat liver cells *in vivo* and *in vitro* is mainly mediated by mannose receptors on sinusoidal endothelial cells. *Biochemical Journal*, 291, 749-755.

Malizia, E., Sarcinelli, L. & Andreucci, G. (1977). Ricinus poisoning: a familiar epidemy. *Acta Pharmacol Toxicol (Copenh)*, 41 Suppl 2, 351-61.

Meldrum, W. (1900). Poisoning by castor oil seeds. *BMJ*, 8, 317.

Mistry, D. (2004). Analysis of the fundamentals. *Globoil India 2004: The Solvent Extractors Association of India*.

Morlon-Guyot, J., Helmy, M., Lombard-Frasca, S., Pignol, D., Pieroni, G. & Beaumelle, B. (2003). Identification of the ricin lipase site and implication in cytotoxicity. *J Biol Chem*, 278, 17006-11.

O'Connell, K.P., Menuey, B.C. & Foster, D. (2002). Issues in preparedness for biologic terrorism: a perspective for critical care nursing. *AACN Clin Issues*, 13, 452-69.

Olsnes, S. & Saltvedt, E. (1975). Conformation-dependent antigenic determinants in the toxic lectin ricin. *J Immunol*, 114, 1743-8.

Paddle, B.M. & Porteski, M. (2003). Evaluation of the neutralizing activity of antibodies against ricin in a cytotoxicity assay utilizing human lung epithelial cells. *DSTO Technical Report*, 1-19.

Palatnick, W. & Tenenbein, M. (2000). Hepatotoxicity from castor bean ingestion in a child. *J Toxicol Clin Toxicol*, 38, 67-9.

Pappenheimer, A.M., Jr., Olsnes, S. & Harper, A.A. (1974). Lectins from *Abrus precatorius* and *Ricinus communis*. I. Immunochemical relationships between toxins and agglutinins. *J Immunol*, 113, 835-41.

Ramsden, C.S., Drayson, M.T. & Bell, E.B. (1989). The toxicity, distribution and excretion of ricin holotoxin in rats. *Toxicology*, 55, 161-71.

Rauber, A. & Heard, J. (1985). Castor bean toxicity re-examined: a new perspective. *Vet Hum Toxicol*, 27, 498-502.

Reed, R.P. (1998). Castor oil seed poisoning: a concern for children. *Med J Aust*, 168, 423-4.

Robertus, J.D., Piatak, M., Ferris, R. & Houston, L.L. (1987). Crystallization of ricin A chain obtained from a cloned gene expressed in *Escherichia coli*. *J Biol Chem*, 262, 19-20.

Roy, C.J., Hale, M., Hartings, J.M., Pitt, L. & Duniho, S. (2003). Impact of inhalation exposure modality and particle size on the respiratory deposition of ricin in BALB/c mice. *Inhal Toxicol*, 15, 619-38.

Sandvig, K., Olsnes, S. & Pihl, A. (1978). Binding, uptake and degradation of the toxic proteins abrin and ricin by toxin-resistant cell variants. *Eur J Biochem*, 82, 13-23.

Satpathy, R. & Das, B.B. (1979). Accidental poisoning in childhood. *J Indian Med Assoc*, 73, 190-2.

Sharma, S., Podder, S.K. & Karande, A.A. (1999). Comparative studies on kinetics of inhibition of protein synthesis in intact cells by ricin and a conjugate of ricin B-chain with momordin. *Mol Cell Biochem*, 200, 133-41.

Simmons, B., Stahl, P. & Russell, J. (1996). Mannose receptor-mediated uptake of ricin toxin and ricin A chain by macrophages. *The Journal of Biological Chemistry*, 261, 7912-7920.

Valdivieso-Garcia, A., Clarke, R.C., Rahn, K., Durette, A., Macleod, D.L. & Gyles, C.L. (1993). Neutral Red Assay for Measurement of Quantitative Vero Cell Cytotoxicity. *Appl Environ Microbiol*, 59, 1981-1983.

Vitetta, E.S., Smallshaw, J.E., Coleman, E., Jafri, H., Foster, C., Munford, R. & Schindler, J. (2006). A pilot clinical trial of a recombinant ricin vaccine in normal humans. *Proc Natl Acad Sci U S A*, 103, 2268-73.

Wedin, G.P., Neal, J.S., Everson, G.W. & Krenzelok, E.P. (1986). Castor bean poisoning. *Am J Emerg Med*, 4, 259-61.

Wilhelmsen, C.L. & Pitt, M.L. (1996). Lesions of acute inhaled lethal ricin intoxication in rhesus monkeys. *Vet Pathol*, 33, 296-302.

Zhang, S.Z., Lipsky, M.M., Trump, B.F. & Hsu, I.C. (1990). Neutral red (NR) assay for cell viability and xenobiotic-induced cytotoxicity in primary cultures of human and rat hepatocytes. *Cell Biol Toxicol*, 6, 219-34.

DEFENCE SCIENCE AND TECHNOLOGY ORGANISATION
DOCUMENT CONTROL DATA

1. PRIVACY MARKING/CAVEAT (OF DOCUMENT)

2. TITLE

Time- and Concentration-Dependent Cytotoxicity of Ricin in Human Lung Epithelial Cells

3. SECURITY CLASSIFICATION (FOR UNCLASSIFIED REPORTS THAT ARE LIMITED RELEASE USE (L) NEXT TO DOCUMENT CLASSIFICATION)

Document	(U)
Title	(U)
Abstract	(U)

4. AUTHOR(S)

Sharmaine Ramasamy and David Proll

5. CORPORATE AUTHOR

DSTO Defence Science and Technology Organisation
506 Lorimer St
Fishermans Bend Victoria 3207 Australia

6a. DSTO NUMBER	6b. AR NUMBER	6c. TYPE OF REPORT	7. DOCUMENT DATE
DSTO-TR-2024	AR-013-806	Technical Report	July 2007

8. FILE NUMBER	9. TASK NUMBER	10. TASK SPONSOR	11. NO. OF PAGES	12. NO. OF REFERENCES
2006/1092696	03/009	Defence Health Service	21	57

13. URL on the World Wide Web

http://www.dsto.defence.gov.au/corporate/reports/DSTO-TR-2024.pdf

14. RELEASE AUTHORITY

Chief, Human Protection and Performance Division

15. SECONDARY RELEASE STATEMENT OF THIS DOCUMENT

Approved for public release

OVERSEAS ENQUIRIES OUTSIDE STATED LIMITATIONS SHOULD BE REFERRED THROUGH DOCUMENT EXCHANGE, PO BOX 1500, EDINBURGH, SA 5111

16. DELIBERATE ANNOUNCEMENT

No Limitations

17. CITATION IN OTHER DOCUMENTS Yes

18. DSTO RESEARCH LIBRARY THESAURUS http://web-vic.dsto.defence.gov.au/workareas/library/resources/dsto_thesaurus.htm

Ricin, biological warfare agents, toxins, human lung epithelial cells, cytotoxic

19. ABSTRACT

Ricin, a potent ribosome-inactivating hetero-dimeric protein toxin (66kDa) produced in the seeds of the castor bean plant (Ricinus communis), is a Category B Agent on the Centres for Disease Control (CDC) Select Agent List. Using human small airway epithelial cells, this is the first study to investigate the time- and dose-dependent cytotoxic effects of ricin in a human cell line. Ricin (1-100pM) produced a time- and dose-dependent decrease in small airway epithelial cell survival. Ricin (10 and 100pM) reduced cell survival to 57% and 50% respectively, after 12 hours exposure and to 12% cell survival after 24 hours exposure to the toxin. Washing cells after 1-5 minutes exposure to ricin (0.01-100pM) prevented any significant ricin-induced SAE cell death. However, after washing cells exposed to ricin for 15 or 30 minutes, there was a significant reduction in SAE cell survival (84% and 71%, respectively, for 10pM and 45% and 31%, respectively, for 100pM). The results suggest that the binding of ricin to the cell surface and subsequent intracellular uptake and cell death appears to be irreversible by washing between 5 and 15 minutes following exposure to the toxin. There may be a window of opportunity for treatment, and therefore prevention, of some lung cell death up to 12 hours after aerosol exposure to ricin given that there was some survival of human lung cells after this time of exposure.